THE ANTIGENIC PROPERTIES OF THE CONSTITUENTS OF THE PNEUMONIC EXUDATE.*

SERUM STUDIES IN PNEUMONIA. II.

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In a previous communication,[1] some observations were reported concerning the antigenic properties of normal fibrin and fibrin from patients having pneumonia to normal serum and serum from pneumonia patients. In brief, the main conclusion drawn at this time was that human serum (both normal and pneumonic) sometimes contains antibodies for human fibrin, which, in part, may be responsible for the dissolution of the fibrinous exudate in pneumonia.

The present report is a continuation of the studies suggested by the earlier experiments. Altho there are some points here discussed which lead us from the main trend of the work and which do not have a direct bearing upon conditions in pneumonia, they will be included, since they are interwoven with the methods used in the experiments and may aid in explaining some of the reactions observed. The same methods of securing fibrin and of carrying out the hemolytic tests were used here as in the previous work.

Since only a few specimens of fibrin showed antigenic properties to human serum, several methods to enhance this antigenic power were tried. One was to add varying amounts of a fat or lipoid solution to the fibrin. The fats used were recovered from pneumonic lungs by Dr. Oskar Klotz. The strength of the solution was the same as that used in the Noguchi test (0.3 gm. in 10 c.c. alcohol and ether [9–1], and this diluted 1 in 10 with salt solution). This dilute solution was itself found to be hemolytic in amounts of five drops. It was only slightly anticomplementary and in the presence of some sera proved to be antigenic. This antigenic property of the fats was not constant for pneumonic, normal, or positive syphilitic serum.

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[1] *Jour. Infect. Dis.*, 1913, 13, p. 69.

No difference in the antigenic power of fibrin with and without the addition of the fats was found in the presence of 25 different sera, 17 being from pneumonia and 8 from normal individuals. The anticomplementary power was greatly increased by the presence of both fibrin and fats, as will be shown later.

Thirty-six specimens of fibrin (18 pneumonic and 18 controls) were used in testing the hemolytic property of the powdered fibrin suspended in salt solution. Among the 18 specimens of fibrin from pneumonia patients, 4 were found to be hemolytic. None of the controls showed this power. This power on the part of powdered fibrin to produce hemolysis was very decided when present.

The fatty substances isolated from pneumonic lungs by Dr. Oskar Klotz have been found to be very hemolytic, five drops of the 1–10 dilution in salt solution causing complete hemolysis of 1 c.c. of a 1 per cent suspension of human red cells in three hours. The fibrin suspension used as antigen was found to inhibit this hemolysis by fats when used in fairly large quantities (8–10 drops). There was evidence of a slight variation in the power of the fibrin to inhibit the hemolysis, but this was thought to be within the limits of variance of the strength of the suspension. Several specimens of fibrin from cases of pneumonia which in themselves were hemolytic did not inhibit this hemolysis and even after the extraction of the hemolytic principles by alcohol they did not inhibit the hemolysis in 25 drops (two and one-half times the amount of normal fibrin necessary completely to inhibit hemolysis). Proof of the extraction of the hemolytic principles from the fibrin lay in the finding of a hemolytic power in the alcoholic extract while the residue remained inactive.

In a series of experiments it was noted that human sera, whether normal or pneumonic, in small quantities inhibited the hemolysis by these fats. Consequently some further observations were made whereby it was shown that simple dilution with salt solution did not inhibit the hemolysis by the fats, while serum in sufficient quantities to prevent hemolysis did so regardless of the dilution (1–50). To determine whether heat had any appreciable effect upon this antihemolytic quality of the serum, several portions of

a serum were treated at various temperatures—37° C. for 20 hours; 56° C. for 20 hours; 65° C. for 30 minutes; 75° C. for 30 minutes; 90° C. for 30 minutes—and afterward tested. No diminution in the antihemolytic power of the serum was found.

The protein fraction of the serum possessing this inhibitive power was then determined. The method employed by Quinan[1] was used for the fractionation of the serum. Ten cubic centimeters of normal serum (inactivated at 56° C. for 30 minutes) were diluted with 100 c.c. of distilled water. Carbon dioxid was passed through the diluted serum to saturation, causing a white, flocculent precipitate (globulins insoluble in water). This precipitate was washed thoroughly with distilled water and dissolved in 10 c.c. of saline (Solution 1). The washings and filtrate were combined and reduced by evaporation to approximately the original volume. Magnesium sulfate was added to saturation and the whole allowed to stand over night. A white precipitate appeared which was filtered off (globulin soluble in water). This precipitate was washed with a saturated solution of magnesium sulfate and redissolved in a small amount of water. Barium hydroxid was added in excess, causing a heavy white precipitate (barium sulfate and magnesium hydroxid). The precipitate was removed by filtration. The filtrate was saturated with carbon dioxid and absolute alcohol gradually added. A white flocculent precipitate separated out (water-soluble globulin). The precipitate was washed with absolute alcohol, dried with ether, then redissolved in saline (Solution 2).

The filtrate from the magnesium sulfate precipitation was dialyzed in running water for 18 hours. The remaining solution contained the albumins (Solution 3).

Each fraction was added in increasing quantities to constant hemolytic quantities of fat solution and red blood cells. The antihemolytic power of the water-insoluble globulin (Solution 1) was found to be practically equal to that of the whole serum. There was very little or no inhibitive power present in the other two fractions, the water-soluble globulin and albumin.

To determine whether the hemolytic principles were confined to any one fraction of the fats, they were fractionated according

[1] *Univ. of Calif. Pub.* (Pathology), 1903, 1, p. 1.

to the method used by Noguchi and Bronfenbrenner.[1] The results here given of the four fractions include also the tests of their anticomplementary power.

Fraction	Hemolytic	Anticomplementary
1. Ether insoluble—hot alcohol insoluble.....	−	−
2. " " — " " soluble.....	++	−
3. Ether soluble—acetone insoluble.....	+	−
4. " " — " soluble.....	+	−

Since leukocytes form such a large portion of the pneumonia exudate it was thought that they, or their decomposition products, might lead to the production of antibodies in the blood. The leukocytes were obtained from the centrifuged specimens of defibrinated blood from two cases of pneumonia, and from several abscesses and empyemas opened at operation. The organisms present in the pus were the pneumococcus, staphylococcus, or streptococcus. The leukocytes, as well as the pus, were washed thoroughly with salt solution. The leukocytes from the cases of pneumonia were immediately extracted by distilled water at 37° C. The specimens of pus were divided into portions, one being placed in 50 per cent pure glycerol, the other repeatedly extracted with alcohol and dried with ether. When used, the leukocytes were extracted by distilled water over night at 37° C., those from glycerol being thoroughly washed with saline. Chloroform, xylol, or toluol was added to the distilled water and leukocytes to prevent bacterial decomposition. The sediment was thrown down by the centrifuge.

The watery leukocytic extracts obtained in this way varied considerably; some were clear, others quite opalescent, some were hemolytic, others were not. They were not anticomplementary in small amounts and did not serve as complement. When used as antigen no constant results were obtained in regard to pneumonia sera, as is shown by the following results: The leukocytic extracts in the presence of the serum from 5 cases of pneumonia showed binding of complement; in the presence of 14 others there was no binding. In the presence of 9 of the control sera (3 with positive reactions to the Noguchi test) there was binding of complement, and in the presence of 18 other controls (5 with positive reactions to the Noguchi test) there was no binding.

[1] *Jour. Exper. Med.*, 1911, 13, p. 43.

An interesting observation was made in the course of these experiments, in that binding occurred in the presence of all of six sera used in the tests with the extract of the cells from the two cases of pneumonia. Four of the sera were from pneumonia patients and two from controls.

One specimen of leukocytes (from pus) kept in 50 per cent glycerol when washed and resuspended in saline was not anticomplementary in small amounts, nor did it prove antigenic in the presence of one pneumonia and one normal serum. The leukocytic extracts when added to fibrin as antigen did not increase the antigenic power of the fibrin.

The leukocytic residue after extraction by water was washed in saline and used as antigen. This residue did not prove anticomplementary in small amounts. When it was used as antigen, the results were varying. The residue in the presence of one pneumonia serum bound complement; in the presence of eight other pneumonia sera there was no binding of complement; in the presence of six control sera there was binding, and in the presence of eleven other control sera there was no binding of complement. Of the control sera showing binding, one showed a positive and four a negative reaction to the Noguchi test; among those showing no binding, six showed a positive and four a negative reaction to the Noguchi test.

It was found that the residue of the cells which had been kept in 50 per cent glycerol previous to extraction was hemolytic, while that from previously dried leukocytes was not. By subjecting the hemolytic cell masses to the action of alcohol, it was found that the hemolytic principles disappeared and were demonstrated in the alcoholic extract. The residue, after the alcoholic extraction, was anticomplementary when used in large amounts, a fact which is also true for other leukocytic débris.

The nature of the fats from leukocytes was determined in a manner similar to that for the fats from pneumonic lungs. Several hundred cubic centimeters of thick, creamy pus from an abscess were incubated under toluol for several days at 37° C. Considerable change due to digestion occurred, making the pus very much more fluid. The whole was filtered and the remaining thick,

greasy residue was extracted repeatedly with alcohol, then ether. The alcoholic and ethereal extracts were mixed and filtered until a clear solution was obtained. This was evaporated to dryness, leaving a thick, dark brown, fatty residue, which was divided into several portions for study.

One portion was used to test its hemolytic, anticomplementary, and antigenic power. It was found to be very hemolytic and not anticomplementary and served inconstantly as antigen in the presence of some sera.

In a series of experiments corresponding with those carried out with the other fats it was found that normal fibrin in fairly large quantities (5–10 drops) inhibited the hemolysis by the leukocytic fats. Serum was also found to inhibit this hemolysis.

An interesting observation was made when using the fat solution, the fat solution and fibrin, and the fat solution and serum with the hemolytic system. There was no anticomplementary action by the fat solution alone, the fibrin alone, or the serum alone, while increasing amounts of fat solution in the presence of a constant amount (2 drops) of normal fibrin suspension and the hemolytic system showed three reactions in regard to the hemolysis: (1) in which the fibrin and fats were in too small a quantity to inhibit the hemolysis by the hemolytic system; (2) in which the amounts of fibrin and fat solution were sufficient to inhibit completely the hemolysis by the hemolytic system; and (3) in which the fats were in sufficient excess to cause hemolysis by their own action, tho enough fibrin and fat solution were present to inhibit the hemolysis by the hemolytic system. These same reactions occurred in the presence of both normal and pneumonic serum.

Another portion of the leukocyte fats was fractionated in the same manner as the fats from pneumonic lungs and somewhat similar results found in regard to their hemolytic and anti-complementary power.

Fraction	Hemolytic	Anticomplementary
1. Ether insoluble—hot alcohol insoluble.....	−	−
2. " " — " " soluble.....	+	−
3. Ether soluble—acetone insoluble.....	+	+
4. " " — " soluble.....	++	−

These four fractions were tried with a large number of sera in testing their antigenic properties. It was found that an occasional serum was positive with several portions and without regard to the result of the Noguchi test, so that any fraction may prove antigenic in the presence of some sera.

DISCUSSION.

Since the suggestion appeared in the first paper associating the antigenic property of the fibrin with fatty substances, it has been shown in our experiments that by artificial means this antigenic property of the fibrin cannot be increased by subjecting the fibrin to solutions of fatty substances. The addition of certain quantities of lipoid substances to the fibrin suspensions increased the anti-complementary property of each, a point which will be discussed again.

That a few specimens of fibrin from pneumonia patients should prove to be hemolytic is rather curious. This power is evidently due to associated fatty substances, since this property can be removed by extracting the fibrin with alcohol and it is then present in the alcoholic extract. It is perhaps true that all the specimens of fibrin used were accompanied by a greater or less quantity of fats derived, perhaps from the leukocytes, during the process of clotting. The quantity of fat present was much greater in some than the average. Why this quantity of fatty substance bound to the fibrin should be greatly increased in some cases of pneumonia is not clear, tho, if it is derived from the leukocytes, it may be the result of the increased quantity of fatty substances known to occur in the leukocytes during pneumonia and may be merely an adhesion of the fats to the fibrin.

Fatty substances extracted from the body tissues are known to be hemolytic and Lamar[1] has shown the inhibitory effect of serum upon this hemolytic action. That this inhibitive power should be limited to the one fraction of globulins is interesting and is another indication of the activity and importance of the globulin content of blood serum. Heating the serum does not interfere with this power to inhibit the hemolysis by the fats.

[1] *Jour. Exper. Med.*, 1911, 13, p. 1.

Fibrin which in itself does not cause hemolysis will inhibit the hemolysis by fatty substances. It is interesting that a specimen of fibrin from a patient with pneumonia, which had proved to be hemolytic, when extracted by alcohol lost its power to inhibit the hemolysis by the fats. The removal of the associated fats by the alcohol would not effect this change so that a change in the fibrin itself must have been produced unless the alcohol removed substances other than the fats.

The fats isolated by the digestion of a quantity of pus were mainly of leukocytic origin, as the bulk of the pus was mainly made up of leukocytes, there being little serum present. These fats closely resembled those isolated from the pneumonic lungs. They were hemolytic and, in the presence of some sera, antigenic. This antigenic power bore no relation to the reaction to the Noguchi test. When fractionated into four fractions similar to those of the fats isolated from the pneumonic lungs, they proved to have similar properties. When these fractions were used as antigen it was found that any fraction might prove antigenic in the presence of some sera regardless of the reaction of these sera to the Noguchi test. This finding adds to the large number of substances capable of binding complement in the presence of serum.

Hiss and Zinser[1] have pointed out the beneficial effect of leukocytic extracts in the treatment of a number of infections including pneumonia. To what action this beneficial effect is due is not known. More pronounced results were obtained by their administration than by that of leukocytes or immune serum. The occurrence of a reaction to the treatment by extracts suggests the development of an immunity to them. Manwaring[2] showed the presence of bactericidal substances in leukocytic extracts which apparently are different from those in the serum, a point maintained by Schattenfroh[3] and Daeubler.[4] Buchner and Hahn[5] believe the bactericidal substances to be identical with alexine. Petterson[6] showed that the leukocytes and bone marrow are much more bactericidal in an immunized animal than in normal ones and claims that the bactericidal substances are not secreted by the

[1] *Jour. Med. Research*, 1908, 19, p. 321.
[2] *Jour. Exper. Med.*, 1912, 16, p. 249.
[3] Quoted by Petterson.
[4] *Ibid.*
[5] *Ibid.*
[6] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1905, 42, p. 56.

leukocytes but retained in them until injured. From our results it does not appear that leukocytes act as antigen, as we were unable to demonstrate a complementary antibody with any greater frequency in pneumonic sera than in controls.

It is interesting that the extract of the leukocytes from the two cases of pneumonia bound complement in the presence of all the sera (4 pneumonic and 2 control) with which it was used. It may indicate that some constituents other than fats in the leukocytes in pneumonia have a greater affinity than normal for complement or that these elements are increased in this disease. The great outpouring of leukocytes into the lungs would offer a means of attracting more complement into the alveoli where it would aid in the destruction of the infecting organisms and in the removal of the exudate.

The leukocytic extracts varied, no doubt, greatly in their content, but, as Hiss and Zinser have experienced, there is no method of standardizing them nor of controlling their constancy. They have never served as complement and may prove quite hemolytic. They are anticomplementary in large amounts, but the difference between the amount used in the hemolytic tests and that used to cause binding of complement is so great that the binding in the presence of the serum cannot be due to this anticomplementary action alone. In this extraction the fats are left behind so that the constituents of the extracts are, no doubt, principally proteins.

The question of the reactions reported in these papers being due to the summation or accentuation of the anticomplementary power of the reagents has been carefully considered. It is true that the fibrin, leukocytic extracts and residues, and the fatty substances used as antigen in the experiments are in large quantities capable of preventing the co-operative action of complement with amboceptor. In the quantities used there was not sufficient present to cause any appreciable effect upon the hemolysis by complement-amboceptor action. The sera used showed no evidence of anticomplementary power in quantities at least twice as large as those used in the tests. It is true according to Noguchi[1]

[1] *Loc. cit.*

that the addition of one substance to another, both of which are very slightly anticomplementary, causes an increase in the anticomplementary action of the combination of the two. This increased anticomplementary power is very well shown where the addition of the fat solution to fibrin and to serum in proper proportion causes complete inhibition of the hemolysis by complement amboceptor action, neither of the substances proving anticomplementary in amounts much greater than those used in the experiment. If this same phenomenon occurs between the serum and the antigen used in other fixation tests, an endeavor must be made to measure the anticomplementary power of the combination of the two in order to correctly read the true binding of complement by the specific reaction between serum and antigen. In spite of this evidence of marked anticomplementary power of some combinations of antigens and sera, we feel that the reactions under discussion as binding of complement are true instances of that reaction.

A most obvious omission in these reports is the lack of consideration of the causative factor—the pneumococcus. As a word of explanation we would say that, since there is much hesitancy in accepting the pneumococcus as the center around which all the phenomena of crisis and resolution occur, it appeared desirable for us to study some of the phenomena associated with equally prominent constituents of the lung exudate. We have used each of the important constituents of the pneumonic exudate as antigen, alone and in combination with other substances, in an effort to discover a relationship to processes of immunity developed against them. Most of the results have proved negative. The frequency of non-specific binding of complement by the interaction of various antigens and sera is striking and emphasizes the necessity for very careful interpretation of all such reactions and the necessity for thorough control of all factors.

CONCLUSIONS.

1. The addition of fatty substances and leukocytic extracts to fibrin does not increase its antigenic power.

2. Leukocytic extracts, leukocyte residues, fatty substances from pneumonic exudates and from leukocytes (pus cells) cause

non-specific binding of complement in the presence of some sera regardless of the reaction to the Noguchi test.

3. Some specimens of pneumonic fibrin are themselves hemolytic.

4. Fatty substances from pneumonic lungs and from leukocytes are hemolytic, but not anticomplementary in small quantities.

5. Normal fibrin and the non-hemolytic pneumonic fibrin, human serum, normal as well as pneumonic, inhibit the hemolysis induced by these fatty substances.

6. The inhibitive power of the serum is contained in the globulin fraction precipitated by carbon dioxid.

7. The four fractions of the fatty substances from pneumonic lungs and from leukocytes simulate those used as syphilitic antigen studied by Noguchi and Bronfenbrenner in their hemolytic, anticomplementary, and antigenic powers.

8. The leukocytic extracts vary to a considerable degree in their hemolytic, anticomplementary, and antigenic properties. They do not serve as complement.

9. The residues of leukocytes kept in glycerol are hemolytic and the hemolytic principles may be extracted by and demonstrated in alcohol.

10. Extracts of leukocytes from patients with pneumonia are more strongly antigenic than those from pus cells.

11. The hemolytic substances associated with some pneumonia fibrin can be removed by alcohol, the alcoholic extract proving hemolytic.

12. The anticomplementary power of normal fibrin is greatly increased by the addition of the fatty substances used in the experiments.

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